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**Title:** Tolerance to cadmium toxicity and phytoremediation potential of three *Brassica rapa* CAX1a TILLING mutants

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Tolerance to cadmium toxicity and phytoremediation potential of three *Brassica rapa* CAX1a TILLING mutants

**ABSTRACT**

Cadmium (Cd) is one of the most toxic heavy metals that reduces crop productivity and is a threat to all the food chain including human health. Phytoremediation is an environmentally friendly strategy to clean up soil contaminated with heavy metals. Researchers are selecting new varieties with an enhanced capacity for phytoremediation purposes. Three *Brassica rapa* mutants for CAX1 transporter were obtained through TILLING. The objective of this work is to evaluate the tolerance of these mutants to Cd toxicity and its potential for Cd phytoremediation. For this, the mutants and the parental R-o-18 were grown under control and Cd toxicity conditions(100 µM CdCl2) and growth, Cd accumulation and physiological parameters were analyzed. The results show that *BraA.cax1a* mutation provides greater Cd uptake capacity although only *BraA.cax1a-12* would be useful for phytoremediation because it registered more than three-fold the Cd content of R-o-18 and presented greater Cd tolerance. This tolerance could be due to the higher Ca and Mg accumulations, the maintaining of photosynthesis performance, the enhanced ROS detoxification and AsA/GSH and TCA cycles, the higher malate, and GA4 concentrations and the lower ethylene levels. Briefly, this study identifies *BraA.cax1a-12* as a potential mutant for phytoremediation of Cd contaminated soil and identifies possible physiological elements that contribute to this capacity.

**Keywords:** *Brassica rapa*, Cadmium, Organic acids, Oxidative stress, Phytohormones, Phytoremediation

**Abbreviations:** APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; DC, distribution coefficient; GSH, glutathione; GPX, glutathione peroxidase; GR, glutathione reductase; LOX, lipoxygenase; MDA, malondialdehyde; OAs, organic acids; PCs, phytochelatins; SOD, superoxide dismutase; TCA, tricarboxylic acids; TILLING, targeting induced local lesions in genomes

**1. Introduction**

Heavy metals are important environmental pollutants that reduce crop productivity and are a threat to all the food chain including human health. One of the most toxic heavy metals is cadmium (Cd) being also dangerous due to its high mobility and availability for all living organisms. In plants, Cd produces severe toxicity symptoms including chlorosis, reduced growth, and impairment of physiological processes such as photosynthesis, respiration, transpiration and nutrient uptake (Clemens and Ma, 2016). Thus, Cd triggers reactive oxygen species (ROS) accumulation producing oxidative stress (Sandalio et al., 2009). ROS cause membrane destabilization due to lipid peroxidation (Sharma et al., 2012). In addition, ROS impair chlorophylls biosynthesis and photosystems decreasing photosynthetic performance (D’Alessandro et al., 2013). To counteract ROS effects, plants induce antioxidant systems that maintain cellular redox homeostasis (Sharma et al., 2012). On the other hand, another effect of Cd stress is the changes in the concentration and distribution of phytohormones (Hu et al., 2013; Han et al., 2013).Likewise, the application of phytohormones such as indole-3-acetic acid (IAA), gibberellins (GAs) or cytokinins (CKs) could enhance Cd tolerance through a better antioxidant system and reducing Cd accumulation (Bashri and Prasad, 2016;Ahmad et al., 2015).Thus, phytohormones and the interaction among them could be crucial in the defense of plants against Cd stress (Hu et al., 2013; Han et al., 2013).

In order to avoid the toxic effect of Cd, plants increase the production of GSH and phytochelatins (PCs) and organic acids (OAs) such as citrate, malate, and oxalate. Due to their complexing properties, they form metal-organic complexes that mitigate the toxic effect of heavy metals. GSH plays a role in the elimination of H2O2 and lipid peroxides and in the expression of stress-responsive genes (Gill and Tujeta, 2010). Likewise, PCs and OAs act in the detoxification of heavy metal through direct chelation, facilitating its transport and storage in vacuoles and therefore preventing its toxic effect (Grill et al., 2007; Ryan et al., 2001). Thus, PCs and OAs could be useful in phytoremediation programs either because they increase their absorption and transport towards the shoot or because they increase their tolerance by sequestering the metal in vacuoles (Hawrylak-Nowak et al., 2015).

Phytoremediation is an environmentally friendly strategy to clean up soils contaminated with heavy metals (Azevedo et al., 2012). Species such as *Brassica rapa* are suitable for Cd phytoremediation as they tolerate relatively high levels of Cd and accumulate it in a concentration up to 100 ppm (Baker et al., 2000; D’Alessandro et al., 2013). Researchers are generating and selecting new varieties with enhanced phytoremediation capacity through the modification of certain genes and protein expression (Ali-Zade et al., 2010). Potential targets are CAXs transporters (Baliardini et al., 2015). CAXs are a family of Ca2+/H+ antiporters located on plasma and organelle membranes that play a key role in Ca homeostasis and in signalling processes driven by Ca (Pittman and Hirschi, 2016). CAXs modification could have a great impact on Cd tolerance due to Cd and Ca are antagonistic elements, both elements have similar physical features and they compete with each other for their entry into the plants (Tran and Popova, 2014). In addition, Ca can indirectly alleviate the toxic effects of Cd due to its relation to ROS production (Rodríguez-Serrano et al., 2009). Finally, the most important reason is that CAX1 is able to transport Cd inside the vacuole. Thus, in two independent experiments, Shigaki et al. (2005) and Wu et al. (2011) obtained a CAX1 variant named CAXcd with enhanced Cd transport capacity.

TILLING (Targeting Induced Local Lesions in Genomes) technique is a potential tool to obtain new variants of CAX1 transporter. This technique allows the screening of new variations in target genes that may be useful for the development of plants with improved characteristics (Till, 2003). One of these improvements could be an enhanced phytoremediation capacity. Three mutants of *B. rapa* ssp. trilocularis ’R-o-18’ CAX1a transporter were generated using the TILLING technique: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12* (Lochlainn et al., 2011). These mutations change amino acids (AAs) upstream of the N-terminal autoinhibitory domain, but they could change protein conformation and affect CAX1 function (Graham et al., 2014). In addition, it was observed recently that these mutations produced changes in some primary metabolic processes and in Ca accumulation (Navarro-León et al., 2019). These changes could provide tolerance against some stress such as heavy metal toxicity. Therefore, the objective of this work is to evaluate the tolerance of these mutants to Cd toxicity and to evaluate its potential for phytoremediation purposes.

**2. Material and methods**

*2.1. Plant material, growth conditions, and treatments*

Three mutants of *Brassica rapa* L. subsp. *trilocularis* (Roxb.) genotype R-o-18 (*BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56)) and the parent line R-o-18 were employed as plant material for the experiment. Mutant plants were obtained and identified as described by Lochlainn et al. (2011) and Graham et al. (2014)*.*Seeds were sown on filter paper moistened with milli-Q water (18.2 MV cm) in 9 cm Petri dishes. The dishes were sealed with plastic film and incubated in the dark for 1 d at 4ºC before transferring to pots filled with vermiculite. These pots where placed in a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 22/18ºC (day/night) and 14/10-h photoperiod at a photosynthetic photon flux density of 350 µmol m-2 s-1 (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Throughout the experiment the plants received a modified Hoagland’s solution composed of 4 mM KNO3, 4 mM Ca(NO3)2 • 4 H2O, 2 mM MgSO4 • 7 H2O, 6 mM KH2PO4, 1 mM NaH2PO4 • 2 H2O, 2 μM MnCl2 • 4 H2O, 1 µM ZnSO4, 0.25 μM CuSO4 • 5 H2O, 0.1 μM Na2MoO4 • 2 H2O, 5 µM Fe-chelate (Sequestrene; 138FeG100), and 10 µM H3BO3. This solution, with a pH of 5.5–6.0, was renewed every three days.

*2.2. Experimental* *design and treatments*

Treatments were started 30 days after germination and were kept for 21 days. Plants received two different treatments: Control (without CdCl2) and Cd toxicity (100 µM CdCl2). The applied CdCl2 dose was selected based on the results of a previous experiment with the same plants grown with doses ranging from 0 µM to 250 µM (data not shown). The two factors involved in the experiment were the Cd treatment (C) and the mutant employed (M). The experimental design consisted of a randomized complete block with 12 treatments, eight plants per treatment and three replications each.

*2.3.* *Plant sampling* *and determination of the relative growth rate (RGR)*

Plant leaves were washed with distilled water, dried on filter paper, and weighed for the fresh weight (FW). Half of the leaves from each treatment were frozen at −30ºC for later biochemical assays and the other half of the plant material was lyophilized to measure the dry weight (DW) and the nutrient concentrations. To determine the RGR, leaves from each genotype were sampled immediately before starting the CdCl2 treatment (Ti). The leaves were dried in a forced-air oven at 70 °C for 24 h, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled after 21 days of treatment (Tf). The RGR values were calculated using the equation RGR = (ln DWf − ln DWi)/(Tf − Ti) where T is the time and the subscripts denote the final and initial sampling (i.e. days 0 and 21, respectively, after the beginning of the CdCl2 treatment).

*2.4. Analysis of cadmium, calcium and magnesium concentrations and cadmium* *distribution coefficient (DC)*

Cd, calcium (Ca), and magnesium (Mg) were determined after a sample of 150 mg dry material was subjected to a process of mineralization by wet digestion (Wolf, 1982). Dry leaves were ground and mineralized with a mixture of nitric acid (HNO3)/perchloric acid (HClO4) (v/v) and H2O2 at 30%. From the resulting mineralization, and after the addition of 20 ml of mili-Q H2O, elements concentrations were determined by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA).

DC was calculated as the quotient between Cd concentration in leaves and Cd concentration in roots (Zhu et al., 2003). Cd content was determined as the product of leaf DW and Cd concentration.

*2.5. Pigment concentrations, SPAD chlorophyll value, and PIABS*

Total chlorophyll and carotenoid were extracted in methanol and centrifuged at 5000 × g for 5 min. Thereafter, the absorbance of the supernatant was measured at 664, 648, and 470 nm. The chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) were estimated using the Lichtenthaler equation (Hartmut and Lichtenthaler, 1987). Total Chl was calculated as the sum of Chl *a* and Chl *b*.

The relative chlorophyll content of leaves was measured by using SPAD chlorophyll meter SPAD-502 (Konica Minolta Sensing Inc., Japan). Three measurements were made in each leaf and the average was calculated.

For PIABS measure, plants were adapted to dark for 30 min before measurements using special leaf clip holders that were allocated in each leaf. Chl *a* fluorescence kinetics was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King’s Lynn, Norfolk, UK); the fluorescence transients were induced by red light (650 nm) with 3000 µmol photons m-2s-1 light intensity and recorded by the instrument. Measurements were conducted with six plants of fully expanded leaves at midstem position. Handy PEA software provides the values of performance index for energy conservation from photons absorbed by PSII antenna to the reduction of QB (PIABS) (Strasser et al., 2000).

*2.6.* *ROS detoxification enzyme activities, O2•− and H2O2 concentrations and lipid peroxidation*

Superoxide dismutase (SOD) activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Yu et al. (1998). Catalase (CAT) activity was determined following the consumption of H2O2 at 240 nm (Nakano and Asada, 1981). The O2.− determination was based on the ability to reduce NBT. Optical density was measured at 580 nm (Kubiś, 2008). H2O2 concentration was colorimetrically measured as described by Junglee et al. (2014). Malondialdehyde (MDA) concentration was determined according to Fu and Huang (2001) measuring absorbance at 532 nm. The non-specific absorbance value at 600 nm was obtained to correct the turbidity. MDA concentration was calculated using 155 mM-1 cm-1 as the extinction coefficient.

*2.7. Lipoxygenase (LOX), peroxidases and glutathione reductase (GR) activities*

LOX activity was measured according to Minguez-Mosquera et al. (1993). Ascorbate peroxidase (APX) and glutathione reductase (GR) activities were assayed following Rao et al. (1996). APX activity was determined registering the absorbance change at 290 nm. GR activity was measured after monitoring the oxidation of NADPH at 340 nm for 3 min. Glutathione peroxidase (GPX) activity was measured as described by Elia et al. (2003) with slight modifications using H2O2 as a substrate and the oxidation of NADPH was recorded at 340 nm. The protein concentration of extracts was determined according to the method of Bradford (1976), using bovine-serum albumin as the standard.

*2.8.* *Glutathione (GSH) and* *ascorbate (AsA) assay*

Oxidized GSH (GSSG), and total GSH (reduced GSH + GSSG) were analyzed according to Noctor and Foyer (1998). Reduced GSH levels were estimated as the difference between total GSH and GSSG. The extraction and quantification of total AsA, reduced AsA, followed the method of Law et al. (1983). The dehydroascorbate (DHA) concentration was deduced from the difference between total AsA and reduced AsA. Redox states of AsA and GSH were calculated using the formula: [(Reduced form) X 100]/ [Reduced + Oxidized forms].

*2.9. Extraction and analysis of organic acids*

Malic, citric and oxalic acids were analyzed according to Gómez-Romero et al. (2010) with some modifications. Briefly, 75 mg of freeze-dried and ground leaves were dropped in 1 ml of cold (-20°C) extraction mixture of methanol/water/acetic acid (80/19.5/0.5, v/v/v). The blend was centrifuged (20,000 *g*, 15 min) and re-extracted for 30 min at 4ºC in an additional 1 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridges (SepPak Plus, Waters, USA) and evaporated at 40ºC under vacuum to near dryness. The residue was dissolved in 1 ml water/methanol/acetic acid (94.5/5/0.5, v/v/v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA). 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The analytes were separated using a Zorbax SB-C18 HPLC column (5 µm, 150 x 0.5 mm, Agilent Technologies, Santa Clara, CA, USA), maintained at 30 ºC. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For OAs quantification, calibration curves were constructed for each analyzed component (1, 2.5, 5, and 10 mg l-1).

*2.10.* *Tricarboxylic acids (TCA) enzyme extractions and assays*

Extracts for measuring enzyme activities were made following the method of Li (2000). Citrate synthase (CS) activity was assayed spectrophotometrically by monitoring the reduction of acetyl coenzyme A (CoA) to Co A with 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm (Srere, 1969). Phosphoenolpyruvate carboxykinase (PEPC) activity was measured according to López-Millán et al. (2001). Finally, MDH activity was determined with oxalate as substrate by measuring the decrease in absorbance at 340 nm due to the enzymatic oxidation of NADH (Dannel et al., 1995).

*2.11. Hormone extraction and analysis*

IAA, GAs (GA1, GA3, and GA4), CKs (trans-Zeatin (tZ) and isopentenyl-adenine (iP)), ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and ABA (abscisic acid) were analyzed as in Ghanem et al. (2008) with some modifications. Briefly, 30 mg of homogenized dry material were dropped in 0.5 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20,000 *g*, 15 min) and re-extracted for 30 min at 4ºC in additional 0.5 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, USA) and evaporated at 40ºC under vacuum. The residue was dissolved in 1 ml methanol/water (20/80, v/v) solution using an ultrasonic bath. The dissolved samples were filtered through Millex nylon membrane filters 13 mm diameter of 0.22 µm pore size (Millipore, Bedford, MA, USA). Next, 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. The mass spectra were determined using the Xcalibur software version 2.2. For quantification of plant hormones, calibration curves were constructed for each component analyzed (1, 10, 50, and 100 µg l-1) and corrected for 10 µg l-1 deuterated internal standards. Recovery percentages ranged between 92 and 95%.

*2.12. Statistical analysis*

Data were subjected to a simple ANOVA at 95% confidence. A two-tailed ANOVA was applied to ascertain whether the Cd treatment (C), the mutations (M), or the interaction (C \* M) significantly affected the results. Means were compared by Fisher’s least significant differences (LSD). Linear correlation coefficients between leaf Cd and leaf Ca or Mg concentration were obtained. The significance levels for both analyses were expressed as \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, or NS (not significant). All statistical analyses were carried out using the Statgraphics Centurion XVI software.

**3. Results**

*3.1. Biomass and relative growth rate*

The application of 100 µM CdCl2 to *B. rapa* plants caused a substantial decrease in leaf biomass in comparison to control conditions. However, *BraA.cax1a-12* plants presented higher leaf DW than the other lines. With respect roots DW, Cd affects it in a lesser than leaves. Comparing lines, only *BraA.cax1a-7* presented a lower root DW value in comparison to R-o-18 plants. Finally, under Cd toxicity conditions, *BraA.cax1a-12* registered the highest foliar RGR values, followed by *BraA.cax1a-7* and, lastly, *BraA.cax1a-4* presenting similar value than R-o-18 plants (Table 1).

**Table 1** Leaf and root growth and leaf Ca concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to Cd toxicity.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | Leaf biomass  (g DW plant-1) | Root biomass  (g DW plant-1) | Leaf RGR  (g g-1 day-1) |
| Control | R-o-18 | 0.71±0.04a | 0.19±0.02a | 0.13±0.01c |
|  | *BraA.cax1a-4* | 0.62±0.04a | 0.18±0.01a | 0.14±0.01b |
|  | *BraA.cax1a-7* | 0.65±0.34a | 0.09±0.01b | 0.16±0.01a |
|  | *BraA.cax1a-12* | 0.65±0.03a | 0.13±0.02b | 0.16±0.01a |
|  | *p*-value | NS | \*\*\* | \*\*\* |
|  | LSD0.05 | 0.09 | 0.04 | 0.01 |
| 100 µM CdCl2 | R-o-18 | 0.30±0.02b | 0.14±0.01ab | 0.09±0.01c |
|  | *BraA.cax1a-4* | 0.25±0.03b | 0.10±0.01bc | 0.10±0.01c |
|  | *BraA.cax1a-7* | 0.28±0.03b | 0.08±0.01c | 0.12±0.01b |
|  | *BraA.cax1a-12* | 0.46±0.03a | 0.15±0.02a | 0.13±0.00a |
|  | *p*-value | \*\*\* | \*\* | \*\*\* |
|  | LSD0.05 | 0.07 | 0.04 | 0.01 |
| Analysis of variance | | | |  |
| Cd (C) |  | \*\*\* | \*\* | \*\*\* |
| Mutation (M) |  | \*\* | \*\*\* | \*\*\* |
| C x M |  | \*\* | \*\* | \*\* |
| LSD0.05 |  | 0.06 | 0.03 | 0.01 |

Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.2. Cd concentration, content and distribution coefficient*

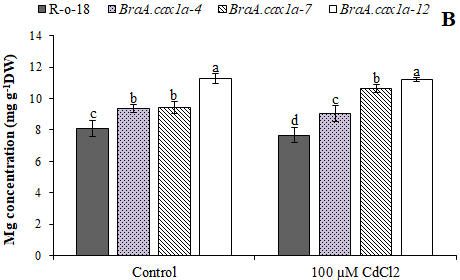
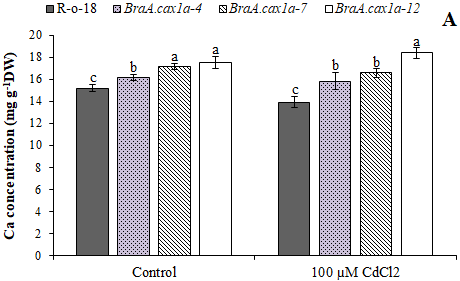
Both *BraA.cax1a-7* and *BraA.cax1a-12* mutations increased Cd concentration and content in leaves, although this increase was greater in *BraA.cax1a-12* plants presenting 104% higher Cd concentration and 212% higher Cd content than R-o-18 plants (Fig. 1A y 1D). On the other hand, root Cd concentration was greater in all mutant lines, being again *BraA.cax1a-12* the mutant that registered the higher levels (Fig. 1B). On the other hand, *BraA.cax1a-12* and *BraA.cax1a-4* plants showed lower DC values in comparison to R-o-18 plants (Fig. 1C).

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**Figure 1** Foliar (A) and root (B) Cd concentration, Cd distribution coefficient (C) and leaf Cd content (D) in *BraA.cax1a* mutants and R-o-18 plants subjected to Cd toxicity. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05).

*3.3. Ca and Mg concentrations*

Cd toxicity did not decrease leaf Ca and Mg concentrations with respect control conditions. However, *BraA.cax1a* plants showed higher Ca and Mg concentrations in comparison to R-o-18 plants under Cd toxicity conditions. Thus, the highest Ca and Mg levels were reached in *BraA.cax1a-12* plants (Fig. 2). In addition, correlation analysis between leaf Cd and Ca concentration showed a positive correlation factor (0.85; P < 0.001) and a similar correlation factor was observed between leaf Cd and Mg concentration (0.83; P < 0.001).

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**Figure 2** Ca (A) and Mg (B) concentrationsin *BraA.cax1a* mutants and R-o-18 leaves of plants grown under Cd toxicity. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05).

*3.4. Pigments concentration and photosynthetic performance*

Under Cd toxicity conditions, *BraA.cax1a-4* and especially *BraA.cax1a-7* plants presented lower SPAD and PIABS values, whereas *BraA.cax1a-12* plants presented no significant differences in comparison to R-o-18 plants. On the other hand, *BraA.cax1a* mutations affected negatively to total Chl concentration. However, *BraA.cax1a* mutants presented higher Chl *a*/*b* ratio values in comparison to R-o-18 plants (Table 2).

**Table 2** SPAD chlorophyll, total Chl concentration, Chl *a*/*b* ratio, and carotenoids concentration in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | SPAD chlorophyll | Total Chl  mg g-1 FW | Chl *a*/*b* | PIABS |
| Control | R-o-18 | 42.44±2.38a | 0.35±0.01a | 2.34±0.02b | 7.74±2.21a |
|  | *BraA.cax1a-4* | 30.08±2.81b | 0.29±0.00b | 2.63±0.10a | 1.91±0.38c |
|  | *BraA.cax1a-7* | 29.52±2.90b | 0.26±0.01c | 2.63±0.07a | 2.43±1.40c |
|  | *BraA.cax1a-12* | 40.06±1.63a | 0.30±0.01b | 2.71±0.09a | 4.95±2.17b |
|  | *p*-value | \*\*\* | \*\*\* | \* | \*\*\* |
|  | LSD0.05 | 2.61 | 0.01 | 0.22 | 2.15 |
| 100 µM CdCl2 | R-o-18 | 37.09±2.21a | 0.43±0.01a | 1.87±0.02b | 5.70±1.98a |
| *BraA.cax1a-4* | 27.54±3.38c | 0.37±0.01b | 2.72±0.16a | 1.07±0.29b |
|  | *BraA.cax1a-7* | 32.32±2.55b | 0.24±0.01d | 2.57±0.06a | 1.51±0.85b |
|  | *BraA.cax1a-12* | 34.94±2.19a | 0.29±0.01c | 2.75±0.14a | 5.43±1.25a |
|  | *p*-value | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 2.62 | 0.02 | 0.37 | 1.49 |
| Analysis of variance | | | | |  |
| Cd (C) |  | \*\*\* | \*\*\* | \* | \* |
| Mutation (M) |  | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| C x M |  | \*\*\* | \*\*\* | \* | NS |
| LSD0.05 |  | 1.81 | 0.01 | 0.21 | 1.26 |

Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.5. Oxidative stress parameters and AsA-GSH cycle*

Cd application caused an increase in O⁠2˙‾ levels and in LOX activity in comparison to control conditions. However, LOX increase was not significant in *BraA.cax1a-12*. With respect to MDA and H2O2 concentrations, when Cd was applied, we only observed an increase in R-o-18 and *BraA.cax1a-7* compared with control plants (Table S1). Comparing between lines, *BraA.cax1a-12* plants presented the lowest O⁠2˙‾, MDA, H2O2 concentrations and the highest LOX, SOD and CAT activities. Likewise, *BraA.cax1a-4* plants also presented lower H2O2 concentration and higher CAT activity in comparison to R-o-18 plants when they were grown with Cd (Fig. 3; Table S1).

**

**Figure 3** Scheme showing the differences between lines grown under Cd toxicity regarding the analyzed elements of the AsA/GSH cycle. Numbers indicate each *BraA.cax1a* mutant: *BraA.cax1a-4* (4), *BraA.cax1a-7* (7), and *BraA.cax1a-12* (12). Upward-pointing black arrows indicate an increment, downward-pointing grey arrows indicate a decrease, and NS indicate non-significant differences with respect R-o-18 plants

*3.6. Ascorbate and glutathione metabolism*

In general, Cd stress reduced the levels of most of the AsA/GSH components in R-o-18. These plants presented lower GR, GPX activities and lower total GSH, GSH, total AsA, AsA and DHA concentrations in comparison to control conditions. In the case of *BraA.cax1a* mutants the reduction in GR activity was lower and presented no significant differences or increments in AsA/GSH cycle components (Table S2; S3; Fig. S1). Comparing between lines, all *BraA.cax1a* presented higher APX, GR and GPX activities, and higher total GSH, GSH concentrations, although *BraA.cax1a-4* reached the highest APX activity and *BraA.cax1a-12* the highest GPX activity. *BraA.cax1a-4* and *BraA.cax1a-12* showed higher total AsA, AsA and DHA concentrations in comparison to R-o-18, although *BraA.cax1a-12* reached the highest concentrations of these parameters (Fig. 3 and S1). Finally, *BraA.cax1a-7* showed lower AsA, AsA and DHA concentrations than R-o-18 but had a higher AsA redox state value (Fig. 3).

*3.7. OAs and TCA cycle*

Regarding malate concentration, the Cd application reduced its concentration in all lines except in *BraA.cax1a-12* that accumulated more of this OA in comparison to the other lines. With respect to citrate, Cd increased its concentration only in *BraA.cax1a-4* and *BraA.cax1a-7* plants and reduced it in *BraA.cax1a-12* plants in comparison to control conditions (Table 3). Comparing between lines, *BraA.cax1a-7* and *BraA.cax1a-12* plants presented lower citrate levels than R-o-18 plants. Finally, oxalate increased in *BraA.cax1a* mutants due to Cd application reaching higher levels than in R-o-18 plants (Table 3).

Cd toxicity caused inhibition of CS activity in comparison to control conditions. However, this inhibition was higher in *BraA.cax1a-7* plants and lower in *BraA.cax1a-12* plants that reached the highest CS activity. No differences between lines were observed regarding MDH activity. In contrast, PEPC activity was lower in *BraA.cax1a-7* plants and higher in *BraA.cax1a-4* and *BraA.cax1a-12* plants, although this last mutant reached the highest PEPC activity (Table 3).

**Table 3** Organic acids concentration andactivities of some TCA cycle enzymes in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Malic acid  (mg g-1DW) | Citric acid  (mg g-1DW) | Oxalic acid  (mg g-1DW) | CS  (ΔAbs mg protein-1min-1) | MDH  (ΔAbs mg protein-1min-1) | PEPC  (ΔAbs mg protein-1min-1) |
| Control | R-o-18 | 12.89±0.70b | 9.09±0.83a | 0.09±0.01a | 0.21±0.02a | 1.13±0.01b | 0.30±0.01b |
|  | *BraA.cax1a-4* | 16.56±1.06a | 5.08±0.55b | 0.06±0.01b | 0.22±0.02a | 0.94±0.02c | 0.36±0.02a |
|  | *BraA.cax1a-7* | 6.52±0.13c | 3.92±0.05b | 0.04±0.00c | 0.21±0.05a | 1.22±0.02ab | 0.10±0.01c |
|  | *BraA.cax1a-12* | 12.74±1.74b | 9.15±0.55a | 0.05±0.01b | 0.19±0.03a | 1.23±0.06a | 0.27±0.02b |
|  | *p*-value | \*\* | \*\*\* | \*\*\* | NS | \*\*\* | \*\*\* |
|  | LSD0.05 | 3.52 | 1.64 | 0.01 | 0.09 | 0.10 | 0.04 |
| 100 µM CdCl2 | R-o-18 | 9.49±0.88b | 8.36±0.66a | 0.05±0.00c | 0.10±0.01b | 0.96±0.02a | 0.29±0.01c |
|  | *BraA.cax1a-4* | 8.95±0.22b | 9.34±0.35a | 0.07±0.01b | 0.10±0.01b | 0.91±0.09a | 0.35±0.01b |
|  | *BraA.cax1a-7* | 5.69±0.09c | 5.59±0.19b | 0.10±0.01a | 0.07±0.01c | 1.00±0.04a | 0.17±0.01d |
|  | *BraA.cax1a-12* | 16.37±0.24a | 6.25±0.01b | 0.08±0.01b | 0.15±0.01a | 0.88±0.12a | 0.40±0.02a |
|  | *p*-value | \*\*\* | \*\*\* | \*\*\* | \*\*\* | NS | \*\*\* |
|  | LSD0.05 | 1.54 | 1.26 | 0.01 | 0.02 | 0.12 | 0.04 |
| Analysis of variance | |  |  |  |  | | |
| Cd (C)  Mutation (M)  D x M  LSD0.05 | | \*\* | NS | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| \*\*\* | \*\*\* | NS | NS | \*\*\* | \*\*\* |
| \*\*\* | \*\*\* | \*\*\* | NS | \*\*\* | \*\*\* |
| 1.77 | 0.95 | 0.01 | 0.04 | 0.07 | 0.03 |

Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*)

*3.8. Phytohormone profile*

Cd affected differently to IAA depending on the line. IAA levels increased in R-o-18 plants, did not show significant changes in *BraA.cax1a-4* plants and decreased in *BraA.cax1a-7* and *BraA.cax1a-12* in comparison to control conditions. However, comparing between lines, IAA concentration only decreased in *BraA.cax1a-7* plants whereas the rest of the lines presented similar IAA values. *BraA.cax1a-12* plants presented the highest GA4 levels, while the rest of the mutants showed similar levels than R-o-18 plants. With respect to total GAs, they were lower in *BraA.cax1a-4* and *BraA.cax1a-7* plants and showed no differences in *BraA.cax1a-12* in comparison to R-o-18 plants. Cd application decreased Ip concentration compared to control plants in all lines. Comparing between lines, iP was lower in *BraA.cax1a-4* and *BraA.cax1a-7* plants whereas tZ concentration was lower in *BraA.cax1a-7* and *BraA.cax1a-12* plants. Thus, total CKs decreased only in *BraA.cax1a-12* plants with respect to R-o-18 plants. Regarding stress-related hormones such as ABA and ACC, Cd application increased greatly ABA concentration in R-o-18 plants and ACC in all lines. Comparing between lines, *BraA.cax1a* present lower levels of these two hormones, presenting *BraA.cax1a-12* the lowest ACC levels (Table 4).

**Table 4** Phytohormones concentration in *BraA.cax1a* mutants and R-o-18 plants grown under Cd toxicity.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | IAA | GA1 | GA3 | GA4 | GAs | iP | tZ | CKs | ABA | ACC |
| Control | R-o-18 | 7.03±0.36c | 0.08±0.02a | Nd | 0.02±0.01a | 0.10±0.01ab | 2.88±0.25a | 203±15ab | 206±15ab | 5.75±1.13ab | 41.77±5.91a |
|  | *BraA.cax1a-4* | 12.61±1.34b | 0.04±0.01ab | Nd | 0.01±0.01a | 0.05±0.01b | 1.45±0.21b | 167±8b | 169±8b | 4.23±0.42b | 33.83±0.67a |
|  | *BraA.cax1a-7* | 10.52±1.74bc | 0.08±0.02a | 0.04±0.01 | 0.01±0.01a | 0.14±0.02a | 1.26±0.27b | 115±1c | 116±1c | 4.31±0.35b | 18.15±1.43b |
|  | *BraA.cax1a-12* | 18.58±1.11a | 0.01±0.00b | 0.07±0.02 | 0.02±0.01a | 0.10±0.02ab | 2.55±0.17a | 231±16a | 234±16a | 7.93±1.71a | 6.28±0.56c |
|  | *p*-value | \*\* | \* |  | NS | \* | \*\* | \*\*\* | \*\*\* | NS | \*\*\* |
|  | LSD0.05 | 4.06 | 0.05 |  | 0.01 | 0.06 | 0.75 | 38 | 38 | 3.46 | 10.02 |
| 100 µM CdCl2 | R-o-18 | 12.94±1.67a | 0.08±0.02 | 0.02±0.01 | 0.01±0.01b | 0.09±0.01a | 3.57±0.44a | 215±15a | 219±14a | 15.28±0.97a | 126.23±1.21a |
| *BraA.cax1a-4* | 10.94±1.56ab | Nd | Nd | 0.02±0.01b | 0.02±0.01b | 1.72±0.06b | 191±6ab | 192±6a | 5.90±0.37bc | 37.50±0.60c |
|  | *BraA.cax1a-7* | 7.54±0.33b | 0.03±0.01 | Nd | 0.02±0.01b | 0.03±0.01a | 1.96±0.17b | 178±9b | 217±26a | 4.78±0.13c | 47.16±2.81b |
|  | *BraA.cax1a-12* | 13.17±0.31a | Nd | 0.02±0.01 | 0.05±0.01a | 0.08±0.02a | 2.96±0.06a | 110±5a | 113±5b | 7.42±0.52b | 18.98±1.19d |
|  | *p*-value | \* |  |  | \*\* | \* | \*\* | \*\*\* | \*\* | \*\*\* | \*\*\* |
|  | LSD0.05 | 3.79 |  |  | 0.02 | 0.04 | 0.87 | 31 | 51 | 1.90 | 5.45 |
| Analysis of variance | | | | |  |  |  |  |  |  |  |
| Cadmium (C)  Mutation (M)  C x M  LSD0.05 | | \* |  |  | \*\* | \*\* | \*\* | \* | \* | \*\*\* | \*\*\* |
| \*\*\* |  |  | \*\*\* | \*\* | \*\*\* | \*\*\* | \* | \*\*\* | \*\*\* |
| \*\* |  |  | \* | \* | NS | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| 2.55 |  |  | 0.01 | 0.03 | 0.50 | 22.50 | 28.95 | 1.81 | 5.24 |

Values are means ± standard error (n=9) expressed in ng g-1 DW and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

**4. Discussion**

*4.1. Plant biomass and RGR*

The negative effect of Cd on plant biomass and growth has been thoroughly studied (D’Alessandro et al., 2013; Srivastava et al., 2015). Thus, in *Brassica juncea* plants the application of 100 μM CdCl2 caused a 30% biomass reduction (D’Alessandro et al., 2013). In the present experiment, we measured roughly a 60% decrease of leaf biomass in all the genotypes except in *BraA.cax1a-12* were the decrease was only 30%. This mutant also presented the highest RGR values (Table 1). Therefore, *BraA.cax1a-12* mutation provides a higher tolerance to Cd toxicity. This higher Cd tolerance could be due to a better Ca homeostasis under Cd stress conditions. Indeed, Ca has been proved as a Cd antagonism mitigating Cd toxic effects (Srivastava et al., 2015).

*4.2. Cd accumulation*

In the present experiment, a**s** it was observed in other similar experiments (Cho et al., 2012 and references therein), the major proportion of applied Cd remained in the root. Therefore, which determines a better phytoremediation efficiency is the capacity to transport the heavy metal to the aerial tissues (Ali-Zade et al., 2010). According to our results, *BraA.cax1a* mutations provide a higher Cd uptake capacity because all mutants accumulated more Cd in roots than parental line R-o-18 (Fig. 1B). Likewise, in the case of *BraA.cax1a-7* and *BraA.cax1a-12,* it provided higher Cd accumulation capacity in the shoot (Fig. 1A). However, *BraA.cax1a* mutation did not provide higher translocation capacity to the shoot as they presented lower DC values than R-o-18 plants (Fig. 1C). Despite this, *BraA.cax1a-12* could be highlighted as the mutant with better phytoremediation efficiency as it presented more than three-fold more Cd content than R-o-18 plants (Fig. 1D). The different Cd accumulation capacity in mutants could be due to the well-known antagonistic relationship between Ca, Mg and Cd (Srivastava et al., 2015) and the effect of *BraA.cax1a* on the accumulation of these elements (Navarro-León et al., 2018). Surprisingly, we did not observe the antagonism between Ca, Mg and Cd. Indeed, we observed positive correlations between Cd and the other cations and *BraA.cax1a-12* was the mutant with higher Ca and Mg concentrations under Cd toxicity (Fig. 2). This could serve as protection against Cd toxicity and could explain in part the higher Cd tolerance in this mutant. Other studies showed that Ca and Mg accumulation could be effective to avoid Cd toxicity (Zorrig et al., 2010; Tran and Popova, 2014). In addition,*BraA.cax1a-7* and *BraA.cax1a-12* through modification in the Ca compartmentalization could induce Cd uptake, probably by reducing Ca cytosol levels which would allow a higher Cd uptake.

*4.4. Pigment and photosynthesis*

Cd causes a negative effect on Chl concentration and in photosynthesis performance (D’Alessandro et al., 2013). However, in the present work, Cd only reduced total Chl concentration in *BraA.cax1a-4* and *BraA.cax1a-7* lines with respect to control conditions (Table 2). Although this reduction was not enough to affect negatively to plant biomass (Table 1). In spite of Chls reduction, *BraA.cax1a* mutations presented a greater Chl *a*/ Chl *b* (Table 2). Chl *a* is principally associated with reaction centers and core antenna proteins of PSII while Chl *b* is principally localized in light-harvesting complexes (LHCII). Therefore Chl *a*/*b* reflects RC/LHCII proportion, when this ratio declines, usually is linked to a reduction of photosynthetic capacity (Nyongesah et al., 2015).Therefore,*BraA.cax1a* under Cd toxicity could favor the conversion of Chl *b* to Chl *a*, in order to maintain Chl *a* levels, and thereby active RCs. In addition, Cd compromise photosynthesis performance inducing conformational changes in LHCII and OEC and interfere with electron transport (D’Alessandro et al., 2013). In our experiment, this negative effect of Cd is clearly observed in R-o-18 plants that presented a reduction in the PIABS parameter in comparison to control conditions. However, this parameter was not affected by Cd in *BraA.cax1a-12* (Table 2). D’Alessandro et al. (2013) proved the protective role of Ca against Cd toxicity. Thus, a greater Ca concentration and homeostasis could protect photosynthetic systems against Cd toxicity in *BraA.cax1a-12* mutant.

*4.5. Oxidative metabolism and AsA/GSH cycle*

One of the most known effects of Cd toxicity is oxidative stress and the subsequent production of ROS and MDA (Sandalio et al., 2009). Likewise, Cd usually inhibits the activity of some antioxidant enzymes such as SOD, APX, and CAT contributing, in turn, to a higher ROS accumulation (Srivastava et al., 2015). As in the majority of studies, we found higher O2·− levels in all plants subjected to Cd stress. However, MDA and H2O2 levels did not increase in *BraA.cax1a-4* and *BraA.cax1a-12* plants and LOX activity did not increase in *BraA.cax1a-12* plants (Fig. 3; Table S1). These two mutants probably possess an improved H2O2 detoxification machinery through higher CAT and APX activities (Fig. 3). On the other hand, *BraA.cax1a-12* presented lower O2·− levels than the other lines which is probably by a higher SOD activity (Fig. 3). High cytosolic Ca levels promote ROS production and decrease of SOD activity under Cd toxicity (Srivastava et al., 2015). Therefore, in *BraA.cax1a-12* plants an enhanced CAX1 activity could reduce ROS levels and allow a greater Cd tolerance.

AsA/GSH cycle is one of the main ways to recycle antioxidants in plants and to eliminate ROS maintaining the reduced state in cells that usually is loss under metal stress (Gill and Tujeta, 2010; Sharma et al., 2012). The effect of Cd application on the AsA/GSH cycle can be either positive or negative depending on the species (Gill and Tujeta, 2010; D’Alessandro et al., 2013; Han et al*.*, 2013). In the present experiment, compared with control conditions, Cd affected negatively to AsA/GSH cycle components in R-o-18 plants but not in *BraA.cax1a* plants (Table S2, S3). Comparing between lines, we observed that *BraA.cax1a-4* and *BraA.cax1a-12* presented an enhanced AsA/GSH cycle under Cd stress in comparison to R-o-18 (Fig. 3). However, this response was greater in *BraA.cax1a-12* plants as they showed higher AsA, GPX activity, and a greater GSH redox state. *BraA.cax1a-7* also appears to have a positive response on GSH part of the cycle although it presented a depletion in AsA levels (Fig. 3, S1; Table S2, S3). These effects of *BraA.cax1a* probably are produced by changes in Ca homeostasis. Thus, it was proved that GSH and the GSH/GSSG ratio is affected by Ca levels as it is reduced under Ca deficiency (Cho et al., 2012, and references therein).Ca also promotes GR and AsA-GSH cycle in Cd treated plants (Srivastava et al., 2015).

*4.6. OAs and TCA cycle*

Several studies proved that OAs such as malate, citrate, and oxalate can bind to heavy metals and increase the metal transport through the xylem and the vacuolar metal sequestration contributing to heavy metal tolerance (Dresler et al., 2014; Clemens and Ma 2016). However, depending on the species analyzed, a different organic acid predominates (Sun et al., 2006; Dresler et al*.*, 2014). In the present experiment, we observed different responses of malate and citrate concentrations depending on the line (Table 3). Thus, in *BraA.cax1a-12* Cd induces malate accumulation what could ease Cd accumulation and tolerance in this mutant. In the rest of the mutants, this malate response was not observed (Table 3) and they accumulated less Cd in shoots (Fig. 1A). Likewise, Han et al. (2006) observed that malate increased Cd uptake in maize plants and enhanced the accumulation in leaves. Regarding citrate, its effects on heavy metal uptake depend on the species (Evangelou et al., 2007). Citrate was not responsible for higher Cd accumulation and tolerance in *BraA.cax1a-12* plants. Finally, with respect to oxalate, there is a positive response of this OA to Cd in *BraA.cax1a* mutants (Table 3). Oxalate could contribute to higher Cd accumulation however it is less important than malate due to its lower concentration in plants (Wang et al., 1991).

In general, Cd toxicity increased TCA cycle enzyme activities (Willick, 2013). Nevertheless,in the present experiment, we observed a negative effect of Cd toxicity on CS activity in all plants in comparison to control conditions (Table 3). Although, *BraA.cax1a-12* plants maintained a higher CS activity which could contribute to higher Cd tolerance (Table 3). Considering the overall TCA activity, we observe three different responses depending on the *BraA.cax1a* mutant. The *BraA.cax1a-4* mutation did not produce changes in TCA enzyme activities except for a slight increase in PEPC activity. *BraA.cax1a-7* affected negatively to TCA cycle activity. In contrast, *BraA.cax1a*-*12* mutation improved CS and PEPC activities (Table 3) which could provide a higher Cd tolerance. Because MDH activity did not increase in *BraA.cax1a*-*12* mutant (Table 3), this mutation could enhance the PEPC pathway as an alternative pathway to produce oxalate.

*4.7. Phytohormone profile*

Hu et al. (2013) and Lin et al. (2018) studies observed that Cd affects negatively to auxin biosynthesis and transport and induce oxidases that degrade auxin and GAs. However, the increase in growth-promoting hormones level appears to be positive in Cd tolerance (Ahmad et al., 2015). In the present study, no clear effect of Cd on IAA concentration is observed because Cd affected differently to each line and all lines presented similar IAA levels except for a decrease in *BraA.cax1a-7* plants (Table 4). Elobeid et al. (2012) showed that IAA affects Cd uptake in plant roots. However, in our study, we did not observe any relationship between Cd uptake and IAA concentration. Regarding GAs, it highlights an increase in GA4 in *BraA.cax1a-12* caused by the Cd application. This mutant presented the highest GA4 levels (Table 4), so GA4 could help to maintain growth in *BraA.cax1a-12* mutant. Likewise, the GA3 application promoted Cd accumulation in *Cannabis sativa* (Ahmad et al., 2015). Thus,GA4 may help to increase also Cd concentration in*BraA.cax1a-12* mutant. On the other hand,Ip concentration considerably increased under Cd stress in comparison to control conditions (Table 4). The same result was observed by Cai et al. (2015) in rice plants. In contrast, Cd decreased CKs concentration only in *BraA.cax1a-12* (Table 4), which in theory could decrease the growth, although this is not produced.

Regarding stress-related phytohormones such as ABA, researchers measured increments in this hormone under Cd toxicity(Han et al., 2013). In contrast, the ABA application is able to reduce Cd accumulation and enhance Cd tolerance in rice seedling (Hsu and Kao, 2005). On the other hand, ethylene, a gaseous hormone synthesized from ACC, increases its synthesis when plants are under stress (Iqbal et al., 2013). Ethylene is able to trigger itself cell mortality induced by Cd as a consequence of a high ROS accumulation (Han et al., 2013). In the present experiment, it was observed a burst of ABA and ethylene levels in R-o-18 plants as a consequence of Cd toxicity (Table 4). However, *BraA.cax1a* plants somehow limit this phytohormone response and keep similar ABA values than in control conditions and a lesser increase of ethylene precursor ACC. With respect to ACC, *BraA.cax1a-12* plants presented lower levels than the other lines (Table 4) which could indicate less stress in this mutant.

**5. Conclusions**

According to the obtained results, all *BraA.cax1a* mutations provide greater Cd uptake capacity but do not increase Cd transport to the shoot. However, *BraA.cax1a-7* and especially *BraA.cax1a-12* mutations provide a greater Cd accumulation in leaves with respect R-o-18. This last mutant registered the greatest phytoextraction capacity with more than three-fold the Cd content of R-o-18. In spite of this, *BraA.cax1a-12* presents greater tolerance to Cd toxicity as it maintains a better growth than the other lines under Cd toxicity. Regarding the physiological response to Cd, it differs depending on the *BraA.cax1a* mutation. *BraA.cax1a-4* presents a similar response as R-o-18 plants although it has higher H2O2 detoxification capacity through AsA/GSH cycle. *BraA.cax1a-7* registered a more negative response because it shows lower AsA and OAs concentrations and lower TCA activity. On the other hand, in *BraA.cax1a-12* the greater Cd tolerance could be due to the higher Ca and Mg accumulations, the maintaining of photosynthesis performance, the enhanced ROS detoxification and AsA/GSH and TCA cycle, the higher malate, and GA4 concentrations and the lower ethylene levels. Briefly, this study identifies *BraA.cax1a-12* as a potential mutant for phytoremediation of Cd contaminated soils and identifies possible physiological elements that contribute to this capacity.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest

**Acknowledgments**

We thank Dr. Martin R. Broadley and Dr. Neil Graham from Nottingham University for providing us the seeds employed in this experiment

**Funding**

This work was supported by the PAI program (Plan Andaluz de Investigación, Grupo de Investigación AGR282) and by a Grant from the FPU of the Ministerio de Educación y Ciencia awarded to ENL [FPU14/01858].

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